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(21) International Application Number: PCT/GB97/01195 (22) International Filing Date: 1 May 1997 (01.05.97) (30) Priority Data: 9609213.5 2 May 1996 (02.05.96) GB (71) Applicant (for all designated States except US): TEPNEL MEDICAL LIMITED [GB/GB]; Toft Hall, Knutsford, Cheshire WA16 9PD (GB). (72) Inventors; and (75) Inventors/Applicants (for US only): MINTER, Stephen, John [GB/GB]; Moor Lodge Farm, Oven Hill Road, New Mills SK12 4QL (GB). OULTRAM, John, Douglas [GB/GB]; 27 Woodsend Road South, Flixton, Manchester M41 6QB (GB). BROWN, Allan [GB/GB]; 6 Westmorland Road, Didsbury, Manchester M20 2TA (GB). (74) Agent: ATKINSON, Peter, Birch; Marks & Clerk, Sussex House, 83-85 Mosley Street, Manchester M2 3LG (GB).		(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG). Published <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
(54) Title: AMPLIFICATION OF NUCLEIC ACIDS (57) Abstract <p>A method of amplifying a nucleic acid sequence, comprises the steps of (a) providing a construct comprised of a closed loop of template nucleic acid, said loop including the sequence to be amplified, and a primer hybridised to the nucleic acid of the loop; and (b) treating the construct (under extension conditions) with a mixture of the four nucleotides and with a polymerising enzyme which is such that (i) it is capable of effecting extension of the primer using the nucleic acid of the loop as a template to form a copy product having a "growing end" at which extension occurs and (ii) nucleic acid complementary to that of the loop and hybridised thereto is displaced therefrom at the "growing end" of the copy product.</p>		

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AMPLIFICATION OF NUCLEIC ACIDS

The present invention relates to the amplification of nucleic acids, i.e. procedures for producing copies of nucleic acid sequences.

As used herein the term "nucleic acid" includes protein nucleic acid (PNA) (i.e. nucleic acids in which the bases are linked by a polypeptide backbone) as well as nucleic acids (e.g. DNA and RNA) having a sugar phosphate backbone.

Various nucleic acid amplification techniques are already known, e.g. the Polymerase Chain Reaction (PCR). However many of these techniques (including PCR) suffer from the disadvantage that various cycles of heating and cooling are required for each amplification reaction. Thus, in a typical amplification reaction, the sequence (in single stranded form) to be amplified is treated with an oligonucleotide capable of hybridising to the sequence at a particular location thereof, the treatment being effected at a temperature (and under other conditions, e.g. buffers etc.) at which the hybridisation will occur. In the next step (which may or may not be effected at the same temperature) a polymerase enzyme is used to extend the oligonucleotide primer (using the original sequence as a template) to produce a strand which is complementary to the original strand and which is hybridised thereto. Subsequently the reaction mixture must be heated to denature the complementary strands and then cooled so that the above described procedure (i.e. primer hybridisation, extension, denaturing) may be repeated. A particular amplification reaction may require many repeats of the cycle before a sufficient quantity of the nucleic acid is generated (e.g. for the purposes of a diagnostic test or for research purposes). The need to "temperature cycle" the reaction many times is a considerable disadvantage.

According to the first aspect of the present invention there is provided a method of amplifying a nucleic acid sequence, comprising the steps of

- (a) providing a construct comprised of a closed loop of template nucleic acid, said loop including the sequence to be amplified, and a primer hybridised to the nucleic acid of the loop; and

(b) treating the construct (under extension conditions) with a mixture of the four nucleotides and with a polymerising enzyme which is such that

(i) it is capable of effecting extension of the primer using the nucleic acid of the loop as a template to form a copy product having a "growing end" at which extension occurs and

(ii) nucleic acid complementary to that of the loop and hybridised thereto is displaced therefrom at the "growing end" of the copy product.

The important features of the first aspect of the present invention are

(1) that the nucleic acid sequence to be amplified is, or is part of, a closed loop, and

(2) the properties of the polymerising enzyme are as defined under (b) above. As described more fully below, this enzyme allows a single stranded copy product comprised of tandem repeats of the loop sequence to be generated, thereby providing an amplification of this sequence.

The enzyme may be referred to as a "strand displacing polymerase" and suitable examples which may be employed in the invention include 9^oN polymerase (Ex-New England Biolabs), Klenow (exo⁻) polymerase, Bst polymerase, Vent (exo⁻) polymerase, and Deep Vent (exo⁻) polymerase.

It is an important advantage of the invention that these agents may be employed at a constant temperature. i.e. the amplification may be effected isothermally.

The reaction may be effected in the solution phase using buffers etc as employed for amplification reactions.

In the simplest embodiment of the invention, the construct has one primer which, for the purposes of explanation, is considered to have an "extension" end from which extension occurs and a "non-extension" end. As well known, the "extension" end will usually be the 3' end of the primer and the "non-extension" end will be the 5' end. Thus the invention will be described by reference to this numbering of the ends but we do not preclude the possibility that (with certain enzymes) extension will proceed in the other direction.

Under the extension conditions employed in the first aspect of the invention, the 3' end of the primer is extended by incorporating the nucleotides and using the loop of the nucleic acid as a template. The "growing end" (i.e. the 3' end of the copy product) continues to "progress" around the loop of the nucleic acid until it reaches the 5' end of this original primer. Owing to the nature of the extension agent employed, the 5' end of the original primer becomes displaced from the loop of nucleic acid. The "growing end" of the copy product continues to copy the nucleic acid of the loop with progressive displacement of the previously formed copy product. As a result, a single strand of copy product of increasing length is produced. This single strand (which is joined to copy product still hybridised to the loop of nucleic acid) comprises tandem repeats of the sequence (or, more correctly, tandem repeats of the complementary sequence) of the nucleic acid of the loop.

It is possible to carry out the method of the invention with more than one primer hybridised to different portions of the nucleic acid loop, in which case, the "growing end" of copy product generated by one primer will progressively displace copy product generated by the other primer and vice versa. This process leads overall to single strands (comprising repeats of the loop sequence) generated from each of the original primers.

The closed loop of nucleic acid may be double stranded and at least one primer may be hybridised to each strand of the loop.

In an advantageous embodiment of the invention, the extension reaction is effected in the presence of a molar excess of at least two solution phase primers, at least one of which is capable of hybridisation to the single stranded copy product. As

explained more fully below with reference to the drawings, this leads to exponential amplification of the loop sequence by a combination of copying and displacement reactions.

This leads to a second aspect of the invention according to which there is provided a method of amplifying a nucleic acid sequence comprising synthesising a linear concatomer of increasing lengths containing tandem repeats of the sequence in the presence of a pair of complementary primers one of which is complementary to the concatomer and in the presence of a mixture of the four nucleotides and a polymerising enzyme which is such that

- (i) it is capable of effecting extension of the primer using the nucleic acid of the linear concatomer as a template to form a copy product having a "growing end" at which extension occurs and
- (ii) nucleic acid complementary to that of the linear concatomer and hybridised thereto is displaced therefrom at the "growing end" of the copy product.

The linear concatomer may be generated by the method of the first aspect of the invention or an alternative technique.

The loop of nucleic acid (which may for example be DNA, RNA or PNA) employed in the first aspect of the present invention may for example be obtained by "circularisation" of a linear length of the nucleic acid to be amplified. One such method of "circularisation" (which provides a further, third, aspect of the present invention comprises the steps of

- (A) preparing a construct in which the ends of a linear nucleic acid are hybridised to respective complementary sequences which are adjacent to each other in an immobilised oligonucleotide whereby the ends of the linear sequence are juxtaposed to each other, and
- (B) ligating said juxtaposed ends.

The "circularised" nucleic acid may then be denatured from the immobilised oligonucleotide for use in the method of the first aspect of the invention. The oligonucleotide is preferably covalently linked to a solid support (e.g. as disclosed in WO-A-9313220).

The construct to which part (A) of the third aspect of the invention refers may be produced in a number of ways. In one method, a linear length of DNA may hybridise to sequences of the oligonucleotide spaced from each other by one or more nucleotides. The portion of the oligonucleotide intermediate the ends of the nucleic acid may then be extended towards each other (using the oligonucleotide as a template) until the ends of the nucleic acid are juxtaposed so that they may be ligated in step (B). Alternatively, the nucleic acid may be hybridised to the oligonucleotide so that the ends of the nucleic acid hybridise in juxtaposed relationship for ligation in step (B).

In addition to the possibility of "circularising" linear nucleic acid for use in the amplification method of the invention, it is also possible to use plasmids, e.g. one which has been genetically engineered to incorporate a sequence of interest.

The invention will be further described by way of example only with reference to the accompanying drawings, in which:

Fig. 1(a)-(i) schematically illustrates one embodiment of the method of the invention;

Fig. 2 illustrates a construct for use in a further embodiment of the invention;

Fig. 3 illustrates a "circularisation" technique in accordance with the third aspect of the invention.

Figs. 4 and 5 illustrate further methods of "circularising" nucleic acids; and

Fig. 6 is an electrophoretogram of the results of Example 2.

Fig. 1 illustrates one embodiment of the present invention with reference to a covalently closed, single stranded length of DNA (hereinafter referred to as "circular" DNA) represented by reference numeral 1. The circular DNA is shown as incorporating a sequence A to which is hybridised a primer B (see Fig. 1(a)).

The method of the invention is effected in the solution phase and involves treatment of the circular DNA 1 under hybridising conditions with a molar excess of a pair of primers A and B (where the former has the same sequence as that depicted for A in the circular DNA 1), a strand displacing polymerase and the four nucleotides dNTP, dATP, dCTP, dGTP. For ease of understanding the primers A and B are shown as being complementary.

In the presence of the displacing polymerase and the four nucleotides, the primer B is extended in the 5' to 3' direction using the circular DNA 1 as a template for copying. Thus, as shown at Fig. 1(b) the extension reaction proceeds to produce a copy strand 2 hybridised to, and extending around, the length of circular DNA 1. This copy strand is depicted as having a "growing end" 3 (represented by the arrow 2 which is that end of the copy product (i.e. the 3' end) at which extension is occurring. As this extension reaction continues, the condition depicted in Fig. 1(c) is reached at which the growing end 3 of the copy strand 2 has extended around the full length of the circular DNA 1 so as to "meet" the original primer B. At this stage, a complementary copy of strand 1 has, in effect, been produced, save that the 5' of primer B is not attached to the "growing end" 3 of the copy product.

The properties of the displacing polymerase are such as to cause the 3' end of the copy product to continue to be extended by using the circular DNA 1 as a template with progressive displacement of that portion of the previously formed copy product encountered by the growing end. This is depicted somewhat schematically in Fig. 1(d).

As the growing end continues to copy around the circular DNA template 1, an increasing length of a single stranded DNA concatomer 4 incorporating copies of primer sequences B is produced, as represented in Fig. 1(f) which depicts the copy strand obtained as the growing end is on its third "circuit" around the circular DNA 1. More particularly, the single strand portion of the copy product is shown as incorporating 2 sequences B (labelled B_0 and B_1). By way of explanation, sequence B_0 is constituted by the original primer displaced after the first circuit of copying by the growing end 3, and sequence B_1 is that formed by the growing end 3 during the

second circuit of copying and displaced at the beginning of the third circuit of copying.

It will therefore be appreciated that copying of the circular DNA 1 continues with production of an increasing length of single stranded concatomer 4 with periodic repeats of sequence B (represented in subsequent drawings as B_0, B_1, B_2, B_n).

The process has been described so far without reference to the function of primers A and B which were used in molar excess.

The sequences B in the concatomer 4 are available for hybridisation with primers A which are then extended using the concatomer 4 as a template. A simplified case is shown in Fig. 1(f) which illustrates annealing of primers A, and extension thereof, to the concatomer 4 illustrated in Fig. 1(e). (For the purposes of clarity the circular DNA 1 is shown in Fig. 1(f) on a smaller scale than in Fig. 1(e)). The primer A_1 (hybridised to B_1) is extended and ultimately causes displacement of the primer A_0 (resulting in the production of a strand A_1-A_0).

With production of an increasing length of concatomer 4, further sites B become available to which primers A can be annealed and extended. For the purposes of illustration, Fig. 1(g) illustrates the concatomer 4 as having 3 sites B (B_0, B_1, B_2). The primer A_2 annealed at B_2 is extended and displaces (into the solution phase) the previously synthesised strand A_1-A_0 producing a further strand (A_2-A_1) as a copy of (and hybridised to) the sequence B_2-B_0 .

In a subsequent stage, as depicted in Fig. 1(h), a further primer A_3 hybridises to a freshly available site B_3 and is extended so as progressively to displace the previously synthesised strand A_2-A_0 . As this latter strand is displaced, its A sites become available for hybridisation with free primers B, e.g. as represented by B_1' which are then extended under the conditions of the reaction.

It will be appreciated that, as the reaction continues, the linear concatomer 4 extends to produce an increasing number of B sites and that each such new site is available for hybridisation (and extension) of a primer A. Thus strands A_n-A_0 synthesised using linear concatomer 4 as a template become of increasing length and produce an increasing number of displaced strands each of which serves for the

generation of further copy strands somewhat as illustrated in Fig. 1(i). It will therefore be appreciated from Fig. 1(i) that an increasingly branched structure is formed (with branches becoming of increasing length) so as to generate greater and greater quantities of the nucleic acids incorporating at least one copy of the sequence of the circular DNA 1 (or its complement) and in many cases a plurality of tandem repeats of such a sequence. The free strands may of course be obtained by subsequent denaturation.

For each circuit of the growing end 3 around the circular DNA 1, the theoretical number of copy sequences produced by the reaction described with reference to Fig. 1 is as shown below:

Circuit	No. of Copy Sequences Produced
1	0
2	1
3	3
4	7
5	15
6	31
7	63
8	127

In the above table, the first circuit is the one which occurs up to the first displacement of the copy strand from the circular DNA 1 and is considered not to provide product. With subsequent circuits, there is a generally exponentially increasing amount of the copy sequence produced due, of course, to the plurality of hybridisation/extension/displacement reactions which are taking place on the linear concatomer and the displaced copies thereof.

The reaction may be effected under isothermal conditions so that the copy product is produced "automatically" by virtue of the presence of the necessary

reagents in the reaction mixture (and appropriate temperature conditions for the polymerase) without the need for temperature cycling.

If it is found that the reaction terminates before sufficient copy nucleic acid is produced, it is possible to produce further construct for amplification by denaturation of the product, dilution to prevent reannealing and ligation.

The procedure described with reference to Fig. 1 employed a construct comprised of the circular DNA 1 and primer B. It is however also possible to employ a construct incorporating more than one hybridised primer, as illustrated in Fig. 2 (to which further reference will be made in the Examples below). The construct of Fig. 2, comprises a loop of double stranded nucleic acid having three primers hybridised to each strand thereof. Extension of the six primers occurs in the direction of the arrow heads.

The circular DNA for use in the method of the first aspect of the invention may be obtained by various techniques and a few examples are given below.

Reference is firstly made to Fig. 3 which is intended to depict the circularisation of a length of DNA 30 shown as having terminal sequences 31 and 32.

The procedure illustrated in Fig. 3 involves use of a solid support 34 having oligonucleotides 35 immobilised thereto (only one shown). The oligonucleotide 35 has two juxtaposed sequences 31' and 32' which are respectively complementary to the sequences 31 and 32 of the DNA 30.

Under hybridising conditions, the DNA 30 becomes immobilised on the oligonucleotide 35 as shown in Fig. 3(b). The ends of DNA 30 may now be ligated (Fig 3(c)) and the resultant circularised product "melted off" the oligonucleotide 35 for use in the method of the invention.

Fig. 4 illustrates an alternative procedure for circularising a length of linear DNA 40 by the use of a "bridging" primer 41 which is complementary to both ends of the DNA 40. The hybridisation of this primer to the ends of the DNA 40 forms a region of double stranded DNA containing a "nick" which can then be ligated. The product is a construct equivalent to that shown in Fig. 1(A).

A further alternative is illustrated in Fig. 5 which involves direct treatment of a dilute solution of linear DNA 50 with T4 RNA ligase which can efficiently ligate the ends of single stranded DNA molecules. The product is circular DNA 51 to which may be hybridised a primer 52 to produce a construct of the type illustrated in Fig. 1(a).

The invention will be illustrated by the following non-limiting Examples.

Example 1

Sequences

RCA003: 5' AGTTCTAACGATTGCTACGA (corresponding to SEQ ID NO: 1)

RCA004: 5' ATATTAAAGGCGAGGTGGAC (corresponding to SEQ ID NO: 2)

RCA005: 5' TCCTGTGTGAAATTGTTATCCAAATGTCCACCTCGCCTTTAAT
ATAGTTCTAACGATTGCTACGAATCTATAATCATGGTCATAGCTGTT
(corresponding to SEQ ID NO: 3)

DOL035: 5' AGCGGATAACAATTTACACAGGAAACAGCTATGACCATGA
TTACGCC (corresponding to SEQ ID NO: 4)

Ligation of Circular Template

An artificial circular template was constructed by ligation of 2.25 pmoles of RCA005, together with 2.25 pmoles of the bridging primer, DOL035, in 100µL total volume of 1 x T4 DNA Ligase Buffer + 1µL of T4 DNA Ligase (400U per µL). The sample was held at 65°C for 5 min and then cooled to 37°C prior to Ligase addition and incubation at 37°C for 60 minutes, followed by 20 minute incubation at 65°C to inactivate the enzyme. Control ligations were also set up containing either, RCA005 alone (-BP i.e. minus Bridging Primer) or No T4 DNA Ligase (-Ligase).

Amplification of Circular Template

The following mix was prepared:

dNTPs (10mM each)	20 μ L
10 x Klenow Buffer	40 μ L
RCA003 (4pmol μ L ⁻¹)	20 μ L
RCA004 (4pmol μ L ⁻¹)	20 μ L
H ₂ O	276 μ L

The mix was dispensed (as 94 μ L aliquots) into 4 x 500 μ L Eppendorf tubes containing 5 μ L of template, which was one of the following:

Control - 10mM Tris-HCL, pH 8.3

- BP (as above)

- Ligase (as above)

+ Ligase (as above)

The mixes were held at 65°C for 5 minutes then allowed to cool to 37°C for 2 minutes, prior to the addition of 1 μ L of Klenow (exo-) DNA Polymerase (5U μ L⁻¹). Incubation was continued at 37°C overnight. Overnight samples (20 μ L) were run on 2% (w/v) agarose gel containing 0.5 μ g ml⁻¹ ethidium bromide. Estimates of the sizes of the bands produced in the +Ligase sample was made using the UVP Geneblot software package, and compared to the sizes predicted by the model;

Estimate	Predicted size
85.4	90
176.4	180
262.0	270
349.0	360

This clearly demonstrates that copying of RCA005 (a 90mer) had taken place.

Example 2**1. Plasmid Multiplex Amplification**

Template: Plasmid pUC19

Primers: A multiplex of six sequence specific primers in two oppositely oriented groups of three, as shown below (and as illustrated in Fig. 2)

DOL006: 5'AGCGGATAACAATTTACACAGGA (corresponding to SEQ ID NO: 5)

DOL034: 5'AACAGCTATGACCATGATTACGCC (corresponding to SEQ ID NO: 6)

ISO002: 5'ATAGGTATGGCAGTGGGTGACGGTGAAAACCTCTGACACAT

(corresponding to SEQ ID NO: 7)

DOL024: 5'CGCCATTCAGGCTGCGCAACTGTT (corresponding to SEQ ID NO: 8)

DOL025: 5'ACAAGCCCGTCAGGGCGCGTCAGC (corresponding to SEQ ID NO: 9)

ISO001: 5'TAATCTTTGGCAGTGGCTTACAACGTCGTGACTGGGAAAAC

(corresponding to SEQ ID NO: 10)

A primer mix was prepared containing all primers at 0.83 pmoles μL^{-1} except DOL006 and DOL025 which were at 0.67 pmoles μL^{-1} .

Reaction Conditions:

10 μL New England biolabs 10 X Vent Polymerase buffer

5 μL Plasmide pUC19 ($\mu\text{g } \mu\text{L}^{-1}$)

5 μL Primer mix

75 μL H₂O

The solution was placed at 95°C for 5 minutes, after which, 5 μL dNTPs (10mM each) and 5 μL 9°N DNA Polymerase (5 U μL^{-1}) were added. The sample was held at 65°C and 20 μL samples were removed after 4 hours. The reaction was terminated immediately, in samples taken, by the addition of 4 μL of agarose loading dye and samples were held at 4°C until all samples were collected. The samples were then analysed by agarose gel electrophoresis in 2.5% (w/v) agarose gel, containing 0.5 $\mu\text{L ml}^{-1}$ ethidium bromide, in 1 x TBE buffer.

The result is shown in the electrophoretogram of Fig. 6 in which the product is in lane 1 and molecular weight marks are in lane 2.

The gel was analysed by GelBolt software and estimates made of the molecular weights of the most prominent bands observed by reference to the known molecular weights of the bands in the marker tracks. The table below shows the predicted bands expected and the corresponding bands observed in the gel. The data show that several of the bands correspond closely to expected sizes and a prominent gel band, which probably represents a doublet of two or more unresolvable bands, is concurrent with a predicted doublet/triplet.

Predicted Value	Estimated Gel Value
500	496
476	NBO
409*	430**
400*	
385*	366
309	320
257	258
233	
157	

NBO - No Band Observed.

* These bands may not be resolved by the gel and may appear as a doublet or triplet band.

** This band appeared to be of higher intensity than other bands and may represent a doublet or triplet band.

Although Fig. 1 above is described with reference to primers A and B which are complementary to each other, with the latter being complementary to a sequence in the circular DNA 1, other arrangements may be used. Thus, for example, one of the solution phase primers may be hybridisable to the concatomer 4 and the other

primer may be hybridisable to a complementary copy thereof without the two primers being complementary to each other.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:

- (A) NAME: TEPNEL MEDICAL LIMITED
- (B) STREET: TOFT HALL
- (C) CITY: KNUTSFORD
- (D) STATE: CHESHIRE
- (E) COUNTRY: UNITED KINGDOM
- (F) POSTAL CODE (ZIP): WA16 9PD

(ii) TITLE OF INVENTION: AMPLIFICATION OF NUCLEIC ACIDS

(iii) NUMBER OF SEQUENCES: 10

(iv) COMPUTER READABLE FORM:

- (A) MEDIUM TYPE: Floppy disk
- (B) COMPUTER: IBM PC compatible
- (C) OPERATING SYSTEM: PC-DOS/MS-DOS
- (D) SOFTWARE: PatentIn Release #1.0. Version #1.30 (EPO)

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

AGTTCTAACG ATTGCTACGA

20

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

ATATTAAAGG CGAGGTGGAC

20

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 90 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

TCCTGTGTGA AATTGTTATC CAAATGTCCA CCTCGCCTTT AATATAGTTC TAACGATTGC 60

TACGAATCTA TAATCATGGT CATAGCTGTT

90

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 48 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

AGCGGATAAC AATTTACAC AGGAAACAGC TATGACCATG ATTACGCC

48

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 24 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

AGCGGATAAC AATTTACAC AGGA

24

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 24 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

AACAGCTATG ACCATGATTA CGCC

24

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 41 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

ATAGGTATGG CAGTGGGTGA CGGTGAAAAC CTCTGACACA T

41

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 24 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

CGCCATTCAG GCTGCGCAAC TGTT

24

(2) INFORMATION FOR SEQ ID NO: 9:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 24 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

ACAAGCCCGT CAGGGCGCGT CAGC

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(2) INFORMATION FOR SEQ ID NO: 10:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 41 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

TAATCTTTGG CAGTGGCTTA CAACGTCGTG ACTGGGAAAA C

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CLAIMS

1. A method of amplifying a nucleic acid sequence, comprising the steps of
 - (a) providing a construct comprised of a closed loop of template nucleic acid, said loop including the sequence to be amplified, and a primer hybridised to the nucleic acid of the loop; and
 - (b) treating the construct (under extension conditions) with a mixture of the four nucleotides and with a polymerising enzyme which is such that
 - (i) it is capable of effecting extension of the primer using the nucleic acid of the loop as a template to form a copy product having a "growing end" at which extension occurs and
 - (ii) nucleic acid complementary to that of the loop and hybridised thereto is displaced therefrom at the "growing end" of the copy product.
2. A method as claimed in claim 1 wherein said construct comprises a plurality of primers hybridised to different portions of the nucleic acid loop.
3. A method as claimed in claim 1 or 2 wherein the loop of template nucleic acid is double stranded.
4. A method as claimed in claim 3 wherein the construct comprises at least one primer hybridised to each strand of the loop.
5. A method as claimed in any one of claim 1 to 4 wherein the extension reaction is effected in the presence of a molar excess of two or more solution phase primers, at least one of which is capable of hybridising to the template loop and at least one of which is capable of hybridisation to the single stranded copy.

6. A method of amplifying a nucleic acid sequence comprising synthesising a linear concatomer of increasing length containing tandem repeats of the sequence in the presence of two or more solution phase primers, at least one of which is capable of hybridisation to the concatomer and another of which is capable of hybridisation to a complementary copy thereof, and in the presence of a mixture of the four nucleotides and a polymerising enzyme which is such that

(i) it is capable of effecting extension of the primer using the nucleic acid of the linear concatomer as a template to form a copy product having a "growing end" at which extension occurs and

(ii) nucleic acid complementary to that of the linear concatomer and hybridised thereto is displaced therefrom at the "growing end" of the copy product.

7. A method as claimed in claim 6 wherein the linear concatomer is synthesised by the method of claim 1.

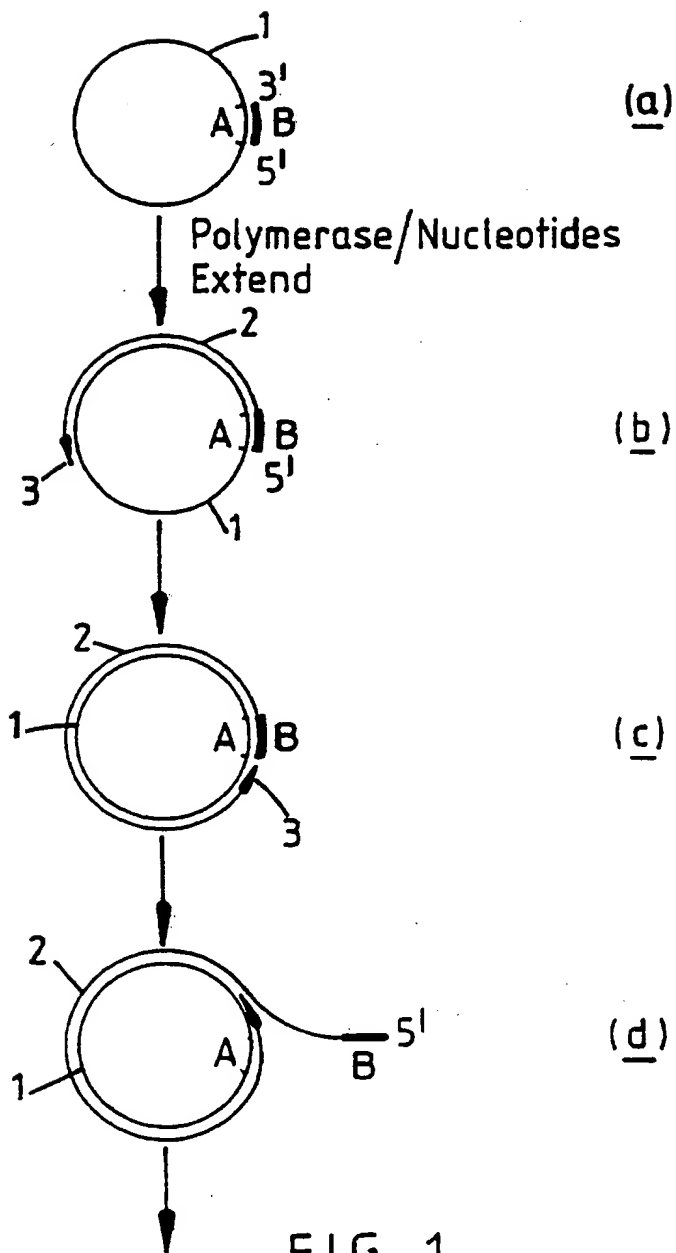
8. A method as claimed in any one of claims 1 to 7 wherein the polymerising enzyme is selected from 9⁰N polymerase, Klenow (exo⁻) polymerase, Bst polymerase, Vent (exo⁻) polymerase, and Deep Vent (exo⁻) polymerase.

9. A method for producing a closed loop of a nucleic acid from a linear length thereof, the method comprising the steps of

(A) preparing a construct in which the ends of a linear nucleic acid are hybridised to respective complementary sequences which are adjacent to each other in an immobilised oligonucleotide whereby the ends of the linear sequence are juxtaposed to each other, and

(B) ligating said juxtaposed ends.

10. A method as claimed in claim 9, wherein the oligonucleotides are immobilised by covalent linkage to solid support.

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(Cont.)

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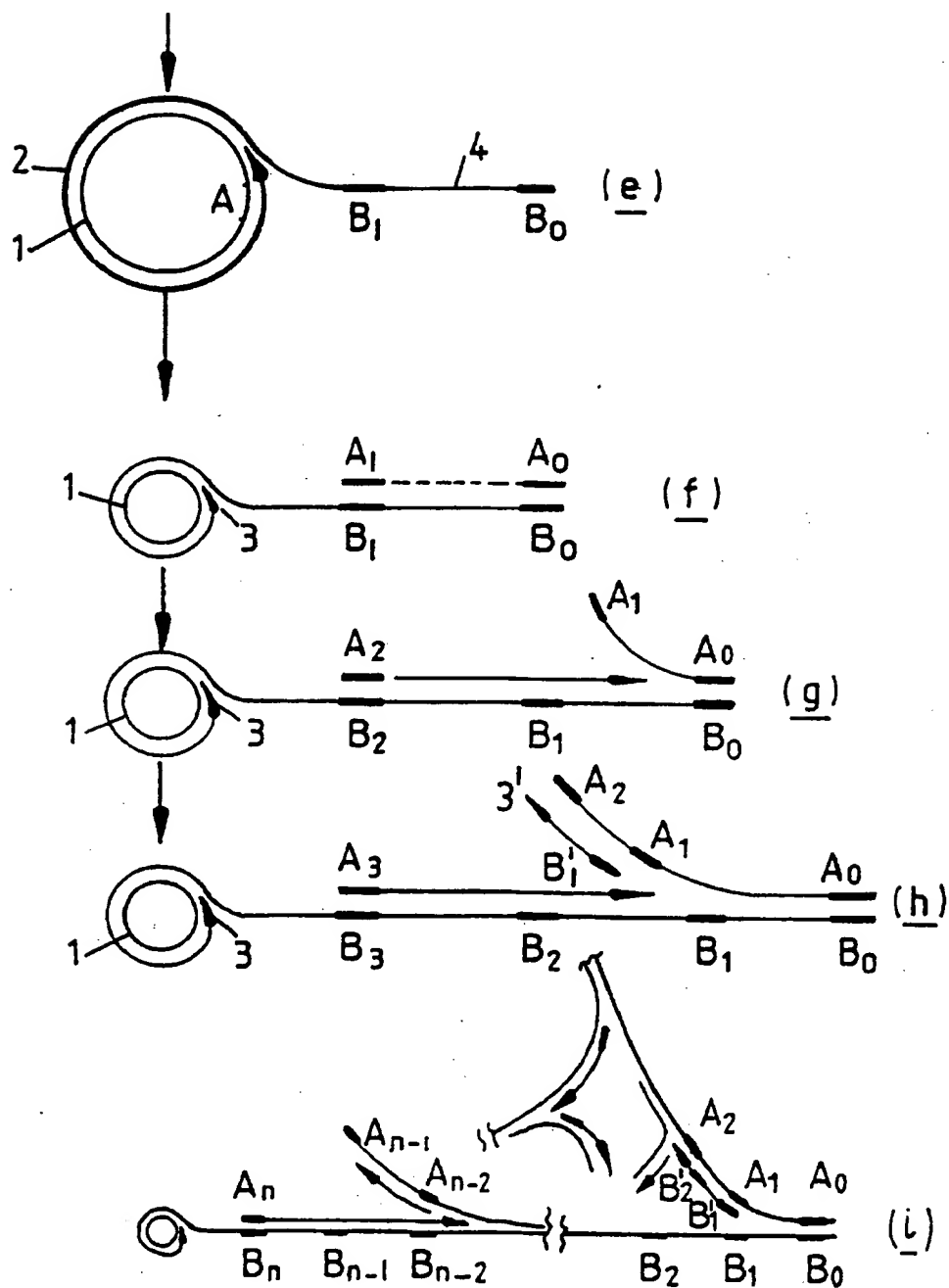


FIG. 1

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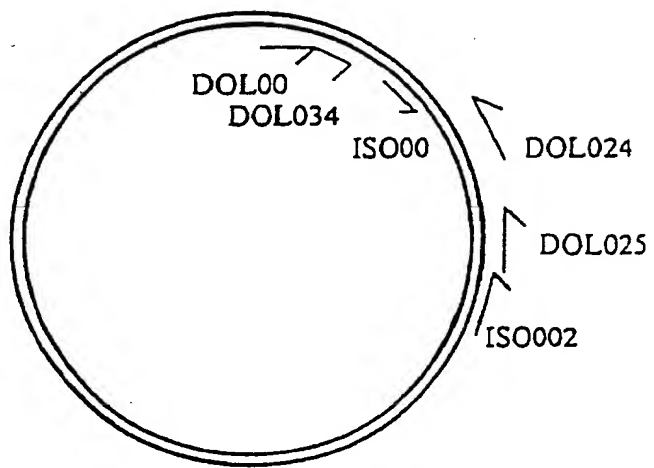
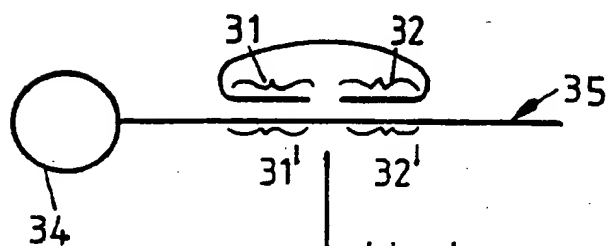
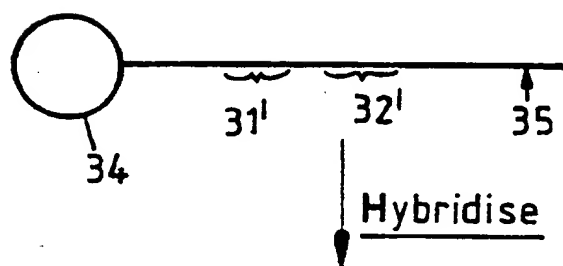
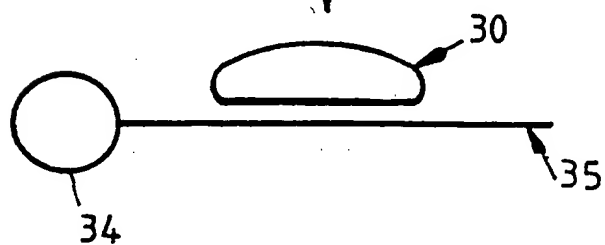


FIG. 2

4-7(a)(b)(c)FIG. 3

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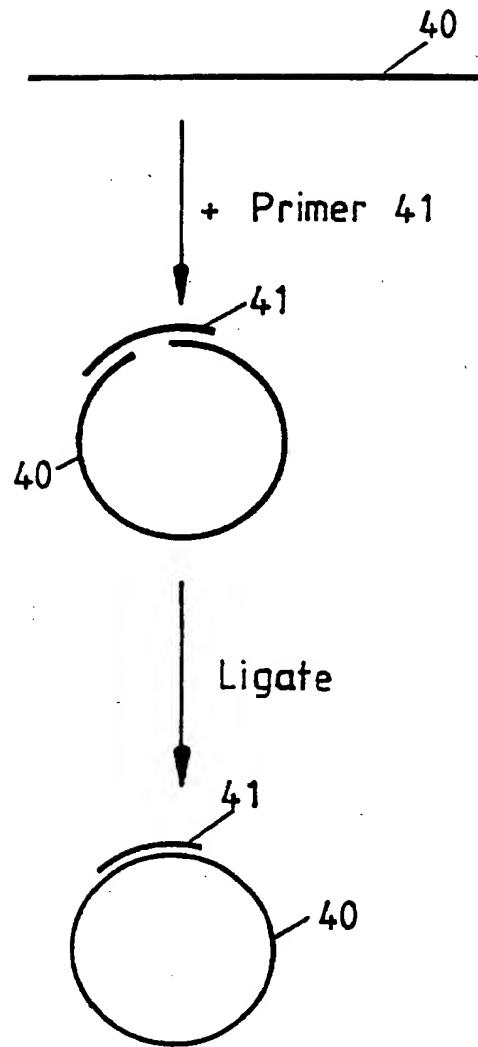
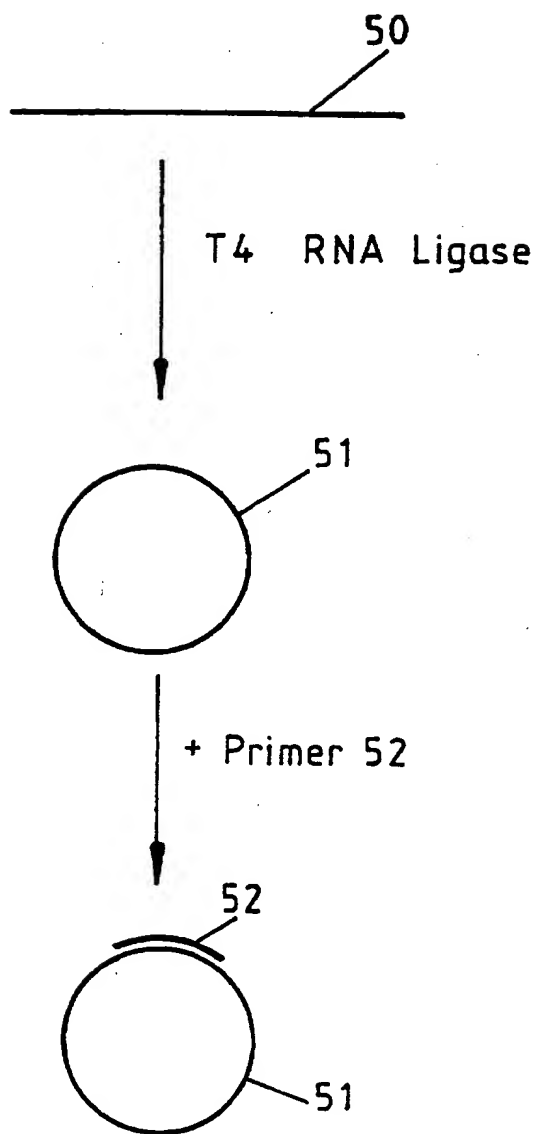


FIG. 4

6-7FIG. 5

7-7



FIG. 6

INTERNATIONAL SEARCH REPORT

Information on patent family members

Internatic Application No

PCT/GB 97/01195

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9201813 A	06-02-92	AU 649066 B AU 8417391 A EP 0542874 A JP 6500014 T	12-05-94 18-02-92 26-05-93 06-01-94
WO 9403630 A	17-02-94	AU 4802793 A CA 2141537 A EP 0658212 A FI 950487 A JP 7509371 T NO 950402 A PL 307341 A AU 676197 B	03-03-94 17-02-94 21-06-95 10-03-95 19-10-95 03-04-95 15-05-95 06-03-97
WO 9719193 A	29-05-97	AU 1024097 A	11-06-97

INTERNATIONAL SEARCH REPORT

Intern. Application No
PCT/GB 97/01195

A. CLASSIFICATION OF SUBJECT MATTER IPC 6 C12Q1/68		
According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELDS SEARCHED Minimum documentation searched (classification system followed by classification symbols) IPC 6 C12Q		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
Electronic data base consulted during the international search (name of data base and, where practical, search terms used)		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 92 01813 A (SYNGENE INC) 6 February 1992 see the whole document ---	1-10
X	WO 94 03630 A (BECKMAN INSTRUMENTS INC ; ADAMS CRAIG W (US); DANIELS DAVID W (US)) 17 February 1994 see claims and figures, and p75, 120 - p81,13 ---	9,10
X	LEWIN: "Genes IV" 1990, CELL PRESS, CAMBRIDGE, MASS. XP002041180 see page 336 - page 338 --- <div style="text-align: right;">-/--</div>	1
<div style="display: flex; justify-content: space-between;"> <input checked="" type="checkbox"/> Further documents are listed in the continuation of box C. <input checked="" type="checkbox"/> Patent family members are listed in annex. </div>		
* Special categories of cited documents:		
<div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p>*A* document defining the general state of the art which is not considered to be of particular relevance</p> <p>*E* earlier document but published on or after the international filing date</p> <p>*L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>*O* document referring to an oral disclosure, use, exhibition or other means</p> <p>*P* document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone</p> <p>*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art</p> <p>*Z* document member of the same patent family</p> </div> </div>		
Date of the actual completion of the international search <div style="text-align: center;">24 September 1997</div>		Date of mailing of the international search report <div style="text-align: center;">07.10.97</div>
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016		Authorized officer <div style="text-align: center;">Müller, F</div>

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INTERNATIONAL SEARCH REPORT

Internat. Application No
PCT/GB 97/01195

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>WALTER N. & STRUNK G.: "Strand displacement amplification as an in vitro model for rolling circle replication: deletion formation and evolution during serial transfer"</p> <p>PROC. NATL. ACAD. SCI., vol. 91, - August 1194 pages 7937-7941, XP002041179 see the whole document</p> <p style="text-align: center;">----</p>	1-10
E	<p>WO 97 19193 A (UNIV YALE) 29 May 1997 see the whole document</p> <p style="text-align: center;">-----</p>	1-10

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